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Sensitive Detection of Human *Caliciviridae* by RT-PCR

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A semi-nested reverse transcriptase-polymerase chain reaction (RT-PCR) was developed for the detection of human *Caliciviridae.* The method was evaluated on faecal samples from patients with gastroenteritis sent to the Norwegian National Institute of Public Health for routine diagnosis by direct electron microscopy (EM). Of 166 samples, 49 were found to contain *Caliciviridae* by EM, while 7 samples contained other viruses. A total of 74 samples was positive by PCR, including all the samples with EM detectable *Caliciviridae,* while specimens containing other agents were negative. Phylogenetic analysis of RNA sequences from 14 Norwegian samples indicated that the viruses present in Norway are evenly distributed when compared to sequences of human *Caliciviridae* from other countries. The PCR primers should therefore be useful for samples from other regions. The phylogenetic analysis did not cluster viruses with a calici-like morphology, but mingled them with sequences from Norwalk-like viruses, indicating that the two morphological types do not represent separate genogroups.

KEY WORDS: gastroenteritis, calicivirus, small round structured virus, RNA sequence, phylogeny

INTRODUCTION

Human *Caliciviridae* are a common cause of food- and water-borne gastroenteritis worldwide [Cubitt, 1989; Hedberg and Osterholm, 1993], and apparently the major cause of outbreaks of viral gastroenteritis in Norway. The family *Caliciviridae* includes two morphologically distinct forms of viruses, the SRSVs (small round-structured viruses), also referred to as Norwalk-like viruses, and the classical caliciviruses [Cubitt, 1987; Caul and Appleton, 1982; Carter and Cubitt, 1995]. Sequence analyses have established that these two distinct morphological types are closely related [Cubitt et al., 1994; Jiang et al., 1993; Lambden et al., 1993]. The term "calicivirus" is frequently used in the literature to describe viral particles with the classical calici-like morphology, but may also imply a member of the *Caliciviridae* family. To avoid confusion we will use the term human *Caliciviridae,* or HuCV.

The *Caliciviridae* possess a single-stranded messenger-sense RNA genome of approximately 7.7 kb, encoding non-structural proteins (ORF1) and a single capsid protein (ORF2). Most *Caliciviridae* have a small open reading frame (ORF3) at the extreme 3′ end [Carter, 1994; Jiang et al., 1993; Lambden and Clark, 1995].

Direct electron microscopy (EM) is the most widely used method for detection of *Caliciviridae.* However, EM requires at least 10^5 to 10^6 viral particles per ml of stool, and two to three days after onset of symptoms the virus concentration tends to decline below detectable levels [Hedberg and Osterholm, 1993; Thornhill et al., 1975].

Several attempts have been made to develop more sensitive assays using immunological or nucleic acid based methods [Hedberg and Osterholm, 1993; Jiang et al., 1995; Khan et al., 1994]. However, designing a reverse transcriptase polymerase chain reaction (RT-PCR) with a superior detection rate compared to EM has been difficult, presumably due to the sequence variations within the human *Caliciviridae* [Jiang et al., 1995]. PCR detection sensitivities in the range of 30–40% compared to EM have been reported [Moe et al., 1994; Norcott et al., 1994]. The aim of the present study was to develop a sensitive and specific assay for the detection of human *Caliciviridae* based on RT-PCR, as well as obtaining sequence information for viruses collected in Norway.

The RNA-dependent RNA polymerase gene has been a region of choice for developing PCR primers for *Caliciviridae,* as it is expected to be reasonably conserved compared to other parts of the genome. Exploiting the increase in available sequence information from this region, we here report a RT-PCR for human *Caliciviridae* that is more sensitive than direct EM.

MATERIALS AND METHODS

Samples

The Department of Virology at the National Institute of Public Health serves as a diagnostic reference labora-

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TABLE I. PCR Primers

Name	Primer sequence and position[a]
CAL1	5′-^{4539}GATACCATTTTGATGCAGATTA^{4560}-3′
CAL1b	5′-^{4541}TACCATTTTGCGGTCGATTA^{4560}-3′
CAL2	5′-^{4716}GA(A/G)GG(G/C)CT(C/G)CCATCTG^{4733}-3′
CAL2b	5′-^{4714}ATGAGGGCCTACCATCTG^{4733}-3′
CAL3	5′-^{4786}CAGAGTGAGGAGCCAGTG^{4786}-3′
CAL4	5′-^{4874}ACAC(A/T)AT(C/T)TCATCATCACCAT^{4854}-3′
CAL5	5′-^{4944}CTTGTTGGTT(G/C)AGGCCATA^{4925}-3′

[a]The numbers refers to the sequence of the Norwalk virus viral genome (m87661). Wobbles are indicated by parentheses.

tory for investigating outbreaks of viral gastroenteritis *in* Norway. Stools were obtained from 166 cases of gastroenteritis in 1994 and 1995. The samples were tested elsewhere for bacterial agents, adenoviruses and rotaviruses by traditional methods. If none of these agents were found, the samples are examined by direct EM [Kjeldsberg, 1986] and RT-PCR. The samples were kept at 4°C until analysis. Aliquots of faecal extracts and stools were later stored at −70°C and −20°C, respectively. A selection of these recent samples, as well as a limited number of older samples, were included in a phylogenetic analysis.

Sample Preparation

Faecal extracts were prepared by suspending 10% v/v of faecal specimens in Hanks' balanced salt solution supplemented with 0.5% bovine serum albumin and antibiotics, and subsequently clarified by centrifugation [Kjeldsberg, 1986]. RNA was extracted from 200 µl of faecal extract by adding 600 µl of the guanidinium thiocyanate containing Solution D [Chomczynski and Sacchi, 1987], 50 µl of 1 M Na-acetate pH 4.6, 600 µl of water saturated phenol, and 100 µl chloroform:isoamyl alcohol (49:1). The aqueous phase was reextracted with 800 µl chloroform:isoamyl alcohol, and vortexed for 10 min at 4°C prior to centrifugation (10 min, 14,000*g* at 4°C). The aqueous phase was precipitated with isopropanol at −20°C for a minimum of one hr. The pellet was washed twice in 70% ethanol and air dried. Recently this extraction protocol has been replaced by the more rapid Trizol (Gibco BRL) method [Simms et al., 1993/94]. The RNA pellets were dissolved in 20 µl distilled water containing 0.5 U/µl RNA guard (Pharmacia) and 5 mM dithiothreitol. A negative control was included with each extraction.

Primers

All the primers tested are listed in Table I. The design of primers was based on RNA dependent RNA polymerase sequences of *Caliciviridae* available in the GenBank database, as well as Norwegian sequences. Two of the present primers (CAL2 and CAL4) make use of the conserved motifs GLPSG and YGDD (Cubitt et al., 1994; Green et al., 1994). The OLIGO computer software (National Bioscience, USA) was used in the construction of primers. The primers were purchased either from R & D Systems (UK) or the DNA Synthesis Core Facility at the Biotechnology Centre of Oslo.

RT-PCR

Five microliters of the RNA extract was reverse transcribed in 25 µl containing 0.16 U/µl AMV Reverse Transcriptase (Promega), the reaction buffer accompanying the enzyme, 1 mM of each dNTP, 1 U/µl RNAguard (Pharmacia), and 5 µM random hexamers (Promega) at 25°C for 10 min and 42°C for 30 min. The samples were heated subsequently to 95°C for 5 min, and cooled to 4°C. A 5µl aliquot was immediately added to 20 µl PCR mixture.

The PCR was run in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.4 mM of each dNTP, 0.4 µM of each primer, 0.04 U/µl of AmpliTaq (Perkin Elmer), and $MgCl_2$ as indicated below. The PCR tubes were transferred to a block preheated to 94°C (2 min), and the reaction run for 30-35 cycles (as well as a final elongation for 2 min at 72°C). In the outer PCR (CAL1–CAL4) the $MgCl_2$ concentration was 3.5 mM and the cycling conditions: 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec. In the semi-nested PCR (CAL2–CAL4) the $MgCl_2$ concentration was 2 mM, and in the nested PCR (CAL2b–CAL3) 2.5 mM. The cycling conditions were the same except that the semi-nested and nested PCRs used an annealing temperature of 55°C. One µl of the outer PCR product was transferred to the semi-nested and nested PCRs. A Perkin Elmer 9600 thermal cycler was used in all the experiments. Negative controls were included throughout the procedure. A diluted PCR product was included as a positive control with each PCR. The PCR products were resolved by electrophoresis in 2% agarose with ethidium bromide, and visualised by UV transillumination. The nested PCR products were run on a 3% gel. A 123 bp DNA ladder (Gibco BRL) was used as a size marker.

Cloning and Sequencing

PCR products were cloned in pCR™II vectors using the TA Cloning kit (Invitrogen). White colonies were dissolved in water and amplified for 20–25 cycles using Lac_L/Lac_H PCR [Berg and Olaisen, 1994]. PCR products from clones containing insert of expected size were used for sequencing. Both Sequenase 2.0 (United States Biochemical) and cycle sequencing (Promega) were carried out according to the manufacturers recommendations, using the M13 forward and reverse (−40) primers flanking the insert. Single-stranded DNA was prepared with biotinylated Lac_H primer and Dynabeads Streptavidin (Dynal, Norway) following the suggested protocol. The results were confirmed by either sequencing two or more independently picked clones (direct sequencing), or by sequencing both strands (cycle sequencing).

Sequence Analysis and Accession Numbers

Sequences were analysed using programs in the GCG Package (version 8, Genetics Computer Group, University of Wisconsin, USA). The Norwegian sequences were aligned with selected sequences of other *Caliciviridae* available in the GenBank with PILEUP. DISTANCES

Fig. 1. HuCV PCR products. **Lanes 1–3:** A faecal sample with a low concentration of HuCV particles examined with respectively the outer PCR, semi-nested PCR and nested PCR. The outer PCR came out negative. **Lanes 4–6;** The same PCRs using a positive control; and lanes 7–9 with a negative control. L = 123 bp ladder.

was used to calculate pairwise sequence similarity (uncorrected), as well as number of substitutions per 100 nucleotides using the Jukes Cantor correction method. The dendrogram was created using the UPGMA method in GROWTREE. The following sequences from the GenBank were included in the analysis, accession numbers are given in parenthesis; Hawaii calicivirus (u07611), Maryland calicivirus (u07612), Bristol virus (x76716), San Miguel sea lion virus (SMSV1) (u15301), Feline calicivirus (FCV) (m86379), Norwalk virus (NV) (m87661), Snow Mountain Agent (SMA) (123831), Rabbit haemorrhagic disease virus (RHDV) (x73046), Southampton Virus (SHV) (107418), HuCV/12C/92/UK (125111), and HuCV/3C/92/UK (125112). The Sapporo sequence is the Sapporo/82 in Matson et al. (1995). The Norwegian sequences were given names using the format "HuCV/ strain designation/year of sampling No." and have been deposited in the GenBank under the accession numbers x89024–89035.

RESULTS

RT-PCR on Faecal Samples

The RT was optimised with respect to primer and nucleotide concentrations. The PCRs were optimised as to annealing temperature, as well as Mg^{2+}- and primer concentrations. With the nested PCR the modified Taguchi optimising method was used [Cobb and Clarkson, 1994]. The various PCR products are shown in Figure 1. The outer primers (CAL1 and CAL4) gave a 335 bp product, the semi-nested primers (CAL2 and CAL4) a 156 bp product, and the nested primers CAL2b (resembling CAL2) and CAL3 a 70 bp product. Two other primers were tested (CAL1b and CAL5), but found to be less useful. The primer positions and sequences are given in Table I.

Of the 166 stool samples analysed by direct EM, 49 were positive for *Caliciviridae,* while another 7 samples were positive for either adeno-, rota-, or astroviruses (Table II). All the 49 samples containing EM detectable *Caliciviridae* were positive in the outer and/or semi-nested PCR. For two samples, however, the RT-PCR had to be repeated a second time to give a positive result.

TABLE II. Results of Direct EM and PCR on Stools from Outbreaks of Gastroenteritis in 1994 and 1995

	HuCV PCR pos	HuCV PCR neg	Sum
HuCV EM pos	49	0	49
Other EM pos[a]	0	7	7
EM neg	25	85	110
Sum	74	92	166

[a]Of these 3 contained astroviruses, 3 rotaviruses and 1 adenovirus.

Of the 117 samples that did not contain EM detectable HuCV, 23% were positive by PCR. The 7 samples found to contain other viruses were all negative in the HuCV PCRs, as were other faecal samples containing agents such as group C rotavirus or Shigella.

So far 20 samples have been tested by both the semi-nested and the more recently designed nested PCR. In some cases the nested PCR produced a stronger band than the semi-nested PCR, but the samples were either positive or negative in both reactions.

Sequence Analysis

The PCR products from 14 samples covering 12 different outbreaks were cloned and sequenced. In eight cases the outer PCR product was used for sequencing, in the remaining six the semi-nested PCR product was sequenced. Alignment of the 120 bp between the primer sites of the shorter products gave nucleotide identities from 60 to 100%. The calculated percentage of substitutions ranged from 0 to 59%. The amino acids sequence identity ranged from 62 to 100%, which implies less variability than would be expected if the nucleotide variation had not been restricted by amino acid conservation. Only the samples from the same outbreaks (HuCV/1c/94/No and 1d, as well as HuCV/1a/95/No and 1b) contained RNA of exactly the same sequence.

Sequence information, from the 12 different Norwegian sequences and selected sequences reported elsewhere, spanning the four important primer sites are shown in Figure 2. The information presented covers most of the relevant variability within presently available HuCV sequences, and the primers should be functional with the viral types shown. The semi-nested primers were positive with the feline calicivirus examined, a strain related to the Sapporo virus.

A phylogenetic tree based on the 120 nucleotides discussed above was created using the UPGMA method (Fig. 3). Beside the Norwegian samples, the analysis included a selection of other *Caliciviridae* sequences. The tree may be divided into 3 HuCV genogroups: Group I represented by the Norwalk virus (NV), group II represented by the Snow Mountain Agent (SMA), and a third group containing human Sapporo-like viruses. The older Norwegian sequences (1976–84) belonged to the group II, while the more recent samples (1990–95) were distributed in both group I and II.

Three other methods for inferring phylogenetic relationships were tested: DNADIST, DNAPARS, and DNAML in PHYLIP version 3.5 [Felsenstein, 1993]. All four methods gave comparable clustering for one strain

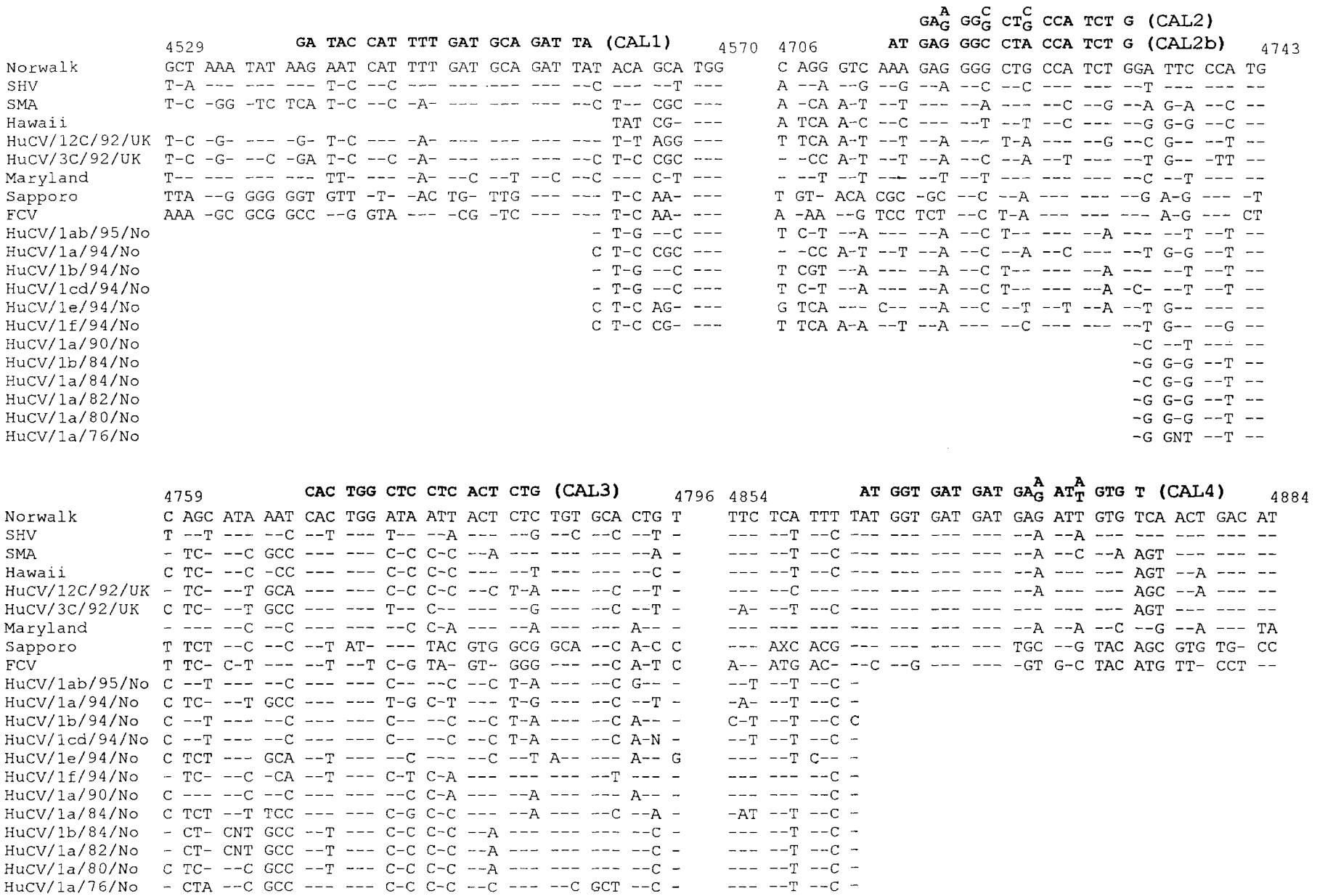
4529 GA TAC CAT TTT GAT GCA GAT TA (CAL1) 4570
4706 AT GAG GGC CTA CCA TCT G (CAL2b) 4743
GA(A/G) GG(C/G) CT(C/G) CCA TCT G (CAL2)
Norwalk GCT AAA TAT AAG AAT CAT TTT GAT GCA GAT TAT ACA GCA TGG C AGG GTC AAA GAG GGG CTG CCA TCT GGA TTC CCA TG
SHV T-A --- --- --- T-C --C --- --- --- --- --C --- --T --- A --A --G --G --A --C --C --- --- --T --- --- --
SMA T-C -GG -TC TCA T-C --C -A- --- --- --- --- --C T-- CGC --- A -CA A-T --T --- --A --- --C --G --A G-A --C --
Hawaii TAT CG- --- A TCA A-C --C --- --T --T --C --- --G G-G --C --
HuCV/12C/92/UK T-C -G- --- -G- T-C --- -A- --- --- --- --- T-T AGG --- T TCA A-T --T --A --- T-A --- --G --C G-- --T --
HuCV/3C/92/UK T-C -G- --C -GA T-C --C -A- --- --- --- --C T-C CGC --- - -CC A-T --T --A --C --A --T --- --T G-- -TT --
Maryland T-- --- --- --- TT- --- -A- --C --T --C --C --- C-T --- - --T --T --- --T --T --- --- --- --C --T --- --
Sapporo TTA --G GGG GGT GTT -T- -AC TG- TTG --- --- T-C AA- --- T GT- ACA CGC -GC --C --A --- --- --G A-G --- -T
FCV AAA -GC GCG GCC --G GTA --- -CG -TC --- --- T-C AA- --- A -AA --G TCC TCT --C T-A --- --- --- A-G --- CT
HuCV/1ab/95/No - T-G --C --- T C-T --A --- --A --C T-- --- --A --- --T --T --
HuCV/1a/94/No C T-C CGC --- - -CC A-T --T --A --C --A --C --- --T G-G --T --
HuCV/1b/94/No - T-G --C --- T CGT --A --- --A --C T-- --- --A --- --T --T --
HuCV/1cd/94/No - T-G --C --- T C-T --A --- --A --C T-- --- --A -C- --T --T --
HuCV/1e/94/No C T-C AG- --- G TCA --- C-- --A --C --T --T --A --T G-- --- --
HuCV/1f/94/No C T-C CG- --- T TCA A-A --T --A --- --C --- --- --T G-- --G --
HuCV/1a/90/No -C --T --- --
HuCV/1b/84/No -G G-G --T --
HuCV/1a/84/No -C G-G --T --
HuCV/1a/82/No -G G-G --T --
HuCV/1a/80/No -G G-G --T --
HuCV/1a/76/No -G GNT --T --
4759 CAC TGG CTC CTC ACT CTG (CAL3) 4796
4854 AT GGT GAT GAT GA(A/G) AT(A/T) GTG T (CAL4) 4884
Norwalk C AGC ATA AAT CAC TGG ATA ATT ACT CTC TGT GCA CTG T TTC TCA TTT TAT GGT GAT GAT GAG ATT GTG TCA ACT GAC AT
SHV T --T --- --C --T --- T-- --A --- --G --C --C --T - --- --T --C --- --- --- --- --A --A --- --- --- --- --
SMA - TC- --C GCC --- --- C-C C-C --A --- --- --- --A - --- --T --C --- --- --- --- --A --C --A AGT --- --- --
Hawaii C TC- --C -CC --- --- C-C C-C --- --T --- --- --C - --- --T --C --- --- --- --- --A --- --- AGT --A --- --
HuCV/12C/92/UK - TC- --T GCA --- --- C-C C-C --C T-A --- --C --T - --- --C --- --- --- --- --- --A --- --- AGC --A --- --
HuCV/3C/92/UK C TC- --T GCC --- --- T-- C-- --- --G --- --C --T - -A- --T --C --- --- --- --- --- --- --- AGT --- --- --
Maryland - --- --C --C --- --- --C C-A --- --A --- --- A-- - --- --- --- --- --- --- --- --A --A --C --G --A --- TA
Sapporo T TCT --C --C --T AT- --- TAC GTG GCG GCA --C A-C C --- AXC ACG --- --- --- --- TGC --G TAC AGC GTG TG- CC
FCV T TC- C-T --- --T --T C-G TA- GT- GGG --- --C A-T C A-- ATG AC- --C --G --- --- -GT G-C TAC ATG TT- CCT --
HuCV/1ab/95/No C --T --- --C --- --- C-- --C --C T-A --- --C G-- - --T --T --C -
HuCV/1a/94/No C TC- --T GCC --- --- T-G C-T --- T-G --- --C --T - -A- --T --C -
HuCV/1b/94/No C --T --- --C --- --- C-- --C --C T-A --- --C A-- - C-T --T --C C
HuCV/1cd/94/No C --T --- --C --- --- C-- --C --C T-A --- --C A-N - --T --T --C -
HuCV/1e/94/No C TCT --- GCA --T --- --C --- --C --T A-- --- A-- G --- --T C-- -
HuCV/1f/94/No - TC- --C -CA --T --- C-T C-A --- --- --- --T --- - --- --- --C -
HuCV/1a/90/No C --- --C --C --- --- --C C-A --- --A --- --- A-- - --- --- --C -
HuCV/1a/84/No C TCT --T TCC --- --- C-G C-C --- --A --- --C --A - -AT --T --C -
HuCV/1b/84/No - CT- CNT GCC --T --- C-C C-C --A --- --- --- --C - --- --T --C -
HuCV/1a/82/No - CT- CNT GCC --T --- C-C C-C --A --- --- --- --C - --- --T --C -
HuCV/1a/80/No C TC- --C GCC --T --- C-C C-C --A --- --- --- --C - --- --T --C -
HuCV/1a/76/No - CTA --C GCC --- --- C-C C-C --C --- --C GCT --C - --- --T --C -

Figure 2.

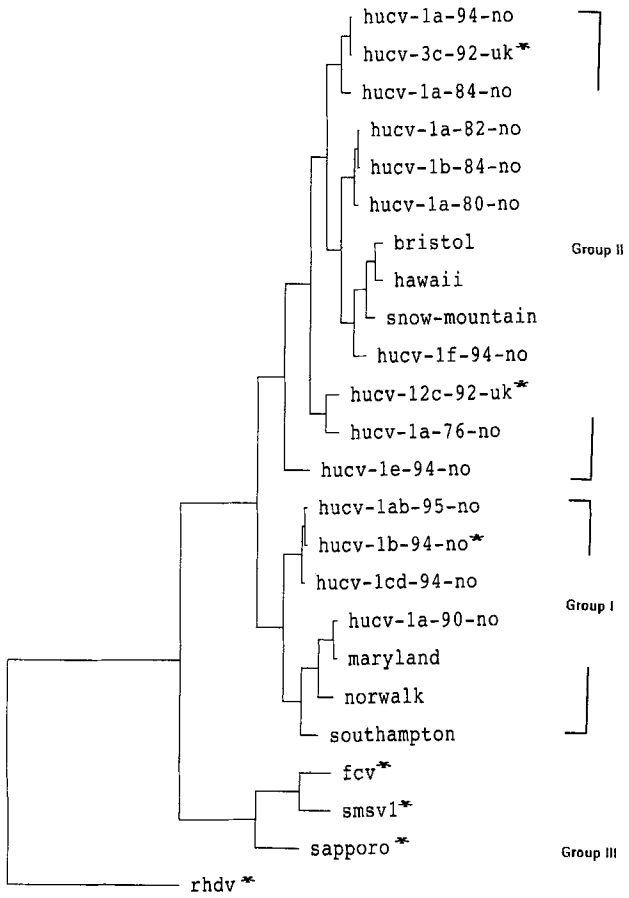

Fig. 3. Phylogenetic relationships of Norwegian and foreign viruses belonging to the *Caliciviridae* family. The phylogenetic analysis was based on 120 nucleotides within the RNA dependent RNA polymerase gene. Abbreviations and GenBank accession numbers are listed under Materials and Methods. An asterisk is used to indicate viruses with calici-like morphology. The Norwegian sequences were named hucv-sample designation-year of sampling-no.

(HuCV/1e/94/No) that grouped within either group I or II. Furthermore, phylogenetic analysis based on the amino acid sequences gave the same main groups.

The two morphological types of *Caliciviridae* are shown in Figure 4. Two of the Norwegian samples contained viral particles with a calici-like morphology, the rest displayed Norwalk-like morphology. One of the calici-like viruses was sequenced (HuCV/1b/94/No). This virus clustered within the group I regardless of the phylogenetic method used. As can be seen in Figure 3, viruses with the calici-like morphology (indicated by an asterisk) are present within all the three major groups.

Fig. 2. Sequences surrounding the primer sites employed in the present study. The primers (or their reverse) are shown above the sequences. The bottom 12 sequences are from the Norwegian samples. Abbreviations and GenBank accession numbers are listed under Materials and Methods. Dashes indicate identity with the Norwalk virus master sequence.

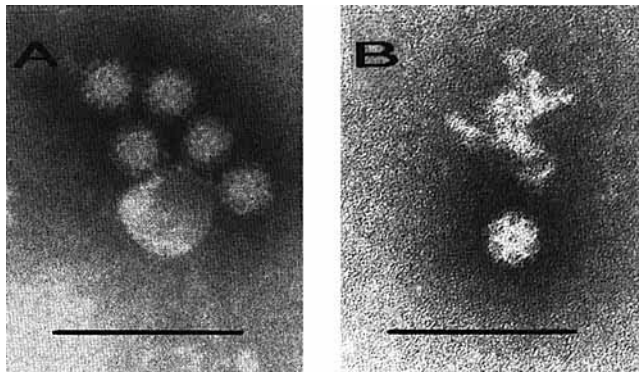

Fig. 4. Electron micrographs of *Caliciviridae*. A: Viruses with the Norwalk-like morphology (also referred to as small round structured viruses). **B:** A virus with the calici-like morphology. The bar represents 100 nm.

TABLE III. Stability of HuCV in Faecal Extracts upon Storage and Freezing as Measured by RT-PCR

Storage conditions	0 day	1 day	7 days	14 days
4°C	+	+	+	−
2× freezing/thawing, 4°C	+	−	−	−
37°C	+	−	−	−
−70°C	+	+	+	+

Viral Stability

In order to indicate the stability of the viral preparation aliquots of a typical sample were stored at either 4°C, 37°C, or −70°C. Other aliquots were frozen at −20°C and thawed to room temperature twice prior to storage at 4°C. The results of testing these aliquots by RT-PCR at various times are shown in Table III. The virus was obviously destabilised by freezing/thawing. Furthermore, the RNA disappeared rapidly at 37°C, and even at 4°C the RNA had limited stability.

DISCUSSION

An RT-PCR is described which is more sensitive than direct EM for the detection of human *Caliciviridae*. Not only were all the EM positive samples positive by PCR, but almost a quarter of the samples suspected of viral gastroenteritis lacking EM detectable viral particles were also positive (Table II). Negative controls, as well as samples shown to contain other agents, were consistently negative. We therefore recommend the semi-nested PCR for routine diagnostic work. The nested PCR was designed at a later stage, partly to supplement the semi-nested PCR, partly to further improve sensitivity. Our experience so far, however, does not indicate that the performance was improved appreciably.

Previously reported PCRs designed for *Caliciviridae* tend to find a substantial number of samples that are positive by EM, but negative by PCR [Willcocks et al., 1993; Moe et al.; 1994; Norcott et al., 1994]. These observations could imply the existence of HuCV with sequences considerably different from those published.

Our primers were designed taking advantage of the increase in available sequence information from this family of viruses. The present result, that all EM positive samples were PCR positive, indicates that most of the *Caliciviridae* causing gastroenteritis in humans contain sequences covered by the proposed primers. This statement is substantiated by the observation that the Norwegian sequences did not cluster, but were distributed among the sequences available from other parts of the world (Fig. 3).

Our experience with RT-PCR on structurally related viruses, such as astro- and rotaviruses, indicates that RNA extraction does not improve the sensitivity. If the preparations are reasonably free of inhibitors, which in the case of faecal extracts may be achieved by dialysis or dilution, the viral genomes are made readily available for the RT-PCR by simply heat-disrupting the viral particles [Jonassen et al., 1995; Grinde et al. 1995]. However, in the case of HuCV this strategy did not give satisfactory results. RNA was therefore extracted from the samples. Initially we used a modified version of the guanidinium thiocyanate protocol described by Chomczynski and Sacchi [1987]. Recently we changed to the less demanding Trizol protocol [Simms et al., 1993/94] and consider that the latter protocol gives equally good results.

Of the 166 samples tested, 87 belonged to outbreaks from where more than one sample was analysed, and where at least one of the samples was HuCV PCR positive. Almost half of these samples (43), however, were negative by PCR. The negative samples were probably taken from a stage in the illness with little shedding of viral particles, and/or the viral particles once present were destroyed due to storage. As indicated in Table III, the stability of these viruses in faecal extracts, as regarding the RT-PCR, is limited. More cases of HuCV gastroenteritis remained probably undiagnosed due to the limited sensitivity of the tests than is the case with other viruses causing gastroenteritis. Samples should preferably be obtained from early stages of illness, kept refrigerated and tested as soon as possible.

The PCR described now takes advantage of conserved islands separated by relatively variable regions. Sequence information obtained from the PCR products may therefore give interesting epidemiological or phylogenetical information. For example, two of the samples sequenced (HuCV/1a/94/No and 1b) were obtained from two outbreaks of gastroenteritis occurring at the same location one month apart. The same source of drinking water was suspected to be the source of virus in both cases. The observation that these sequences were only 66% identical suggests that the two cases originated from separate stocks of virus.

Two observations from the present study indicate that HuCV are dispersed efficiently. One is the lack of clustering of Norwegian sequences when compared to sequences from other countries. In this context it may be significant that the older samples (1976–84) clustered within group II, while the more recent samples (1990–95) displayed more heterogeneity. One would expect the increase in travelling over these years to speed up the spread of viruses. The other observation is that while the Norwegian samples as a whole displayed up to 40% variability in nucleotide sequences, in the two cases where data were obtained from different patients during the same outbreak, the sequences were identical. If this part of the genome mutated rapidly, one might have expected variability even within an outbreak.

The existence of two distinct genogroups, represented by NV (group I) and SMA (group II), have been suggested by several studies [Ando et al., 1994; Lew et al., 1994; Green et al., 1994; Wang et al., 1994]. The viruses tend to divide into the same two genogroups whether sequences from the polymerase region or the capsid protein gene are analysed [Lew et al., 1994; Green et al., 1994, 1995]. Recently, however, viruses with calici-like morphology containing sequences closer to feline caliciviruses than to either of the groups mentioned above have been reported to infect humans [Jiang et al., 1995; Matson et al., 1995]. These viruses may represent cases of transmission from animals to man. One of these group III sequences, Sapporo, was included in our comparison. We do not know whether group III strains will be positive in the present RT-PCR, but a related feline calicivirus was positive. According to the sequences shown in Figure 2, the primers would be expected to be equally functional with the Sapporo strain as with the feline virus.

Most HuCV observed in human faeces are of the Norwalk-like morphology. Viruses with a calici-like morphology are seen only rarely in humans. Recently, however, Cubitt et al. [1994] reported on three samples with calici-like viruses that belonged to the genogroup II. We now report a virus with calici-like morphology belonging to group I. The two morphological types are thus not likely to represent separate phylogenetic clades. The four sequences belonging to calici-like viruses are all closely related to sequences of viruses with Norwalk-like morphology. At least this is the case when comparing sequences of the polymerase gene, sequences of the capsid protein are not available for these samples. The difference in appearance may therefore be due to chemical or physical factors, as suggested by Cubitt et al. [1994]. The observation that certain animal caliciviruses are degraded by adverse physical conditions and/or proteolytic enzymes to smaller and smoother particles may indicate so [Hillman et al., 1982; Moussa et al., 1992]. Alternatively, the morphology may be related to actual differences in genotype, presumably in the capsid protein, but if that is the case, the data suggest that the required genetic change has occurred on more than one occasion.

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